COMPARATIVE EFFICACY OF SELECTED CRYOGENIC PROTECTIVE AGENTS FOR THE PRESERVATION OF BOVINE SPERMATOZOA

by

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INTRODUCTION

Artificial breeding has become an important tool for perpetuating the genetic material of selected males and preventing the spread of breeding diseases. In countries like the United States where 37 percent of the dairy cow numbers are bred artificially, many purebred herds are using artificial breeding. In 1962 more than half of the registrations in all purebred dairy cattle registry associations have been from artificially conceived calves (25, 27, 28, 46, 54). In developing countries like India, it has become the national policy to adopt artificial breeding as an economic method of upgrading the vast numbers of nondescript cattle and buffaloes. Increased attention is being paid to artificial breeding in successive five year plans. The results in the past 12 years have encouraged some of the Indian State Governments to start centralized liquid semen supply centers to meet the semen requirements of the administrative districts.

Since an increasing number of studs are using frozen semen in the United States, it is logical to expect the centralized liquid semen supply centers of the Indian State Governments to switch from liquid semen to frozen semen in the coming decade to facilitate sire evaluation and progeny testing. All these factors call for continued investigations for evolving better techniques of freezing and storing bovine semen.

An evaluation of the present status of freezing of bovine semen discloses that glycerol has been the only practical protective agent for storage of bovine spermatozoa at -79°C or -196°C (45, 49, 54, 56, 58, 62, 63). Although glycerol has proved to be satisfactory for the preservation of spermatozoa at low temperatures (-79°C or -196°C) there are criticisms. Equilibration requires 12-18 hours. It is toxic to sperm at levels above 7 percent, average survival of sperm after freezing is 50 percent and it interferes with the action of streptomycin on vibrio fetus (16, 29, 35, 45, 46, 48, 54, 58, 62).

Investigations with respect to the mode of action of cryogenic protective agents have led to the proposal that other neutral, nontoxic substances of low molecular weight having high solubility in aqueous electrolyte solutions and the ability to permeate the living cells rapidly, might also prove satisfactory or even superior to glycerol (30, 31, 32, 54, 58). Attempts have been made in the last decade to explore the protective capacity of many substances such as ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide and pyridine-N-oxide for erythrocytes, bone marrow cells, mitochondria, endocrine tissue, embryo cells and spermatozoa for low temperature preservation. However, no details of comparative studies are available in most instances (9, 15, 24, 30, 32, 39, 45, 49, 54, 55, 56, 58, 62, 63). The conditions and media used with some of these agents were not suitable for spermatozoa preservation in commercial processing and storage of bovine spermatozoa. For example, a mixture of 0.16 M sodium chloride solution and egg yolk citrate, and Ringer's solution were the basic media used for some semen preservation studies (32, 49, 56). Further investigation of the aforementioned possible cryogenic protective agents using extenders known to be suitable for a long time, low temperature semen storage, may lead to freezing procedures that could conserve either time or semen. Development of procedures for a cryogenic protective agent that would result in higher survival rates and/or require less equilibration time than glycerol, would be a meaningful contribution to the artificial breeding industry.

The purpose of this study was to investigate the cryogenic protective efficacy of ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide and pyridine-N-oxide compared to glycerol, for the preservation of bovine spermatozoa using liquid nitrogen procedures.

REVIEW OF LITERATURE

The harmful effects of freezing living cells have created many problems in the long time storage of bovine spermatozoa (54, 55).

The theory that traumatic injuries, caused by extracellular and intracellular crystallization, which result in death of cells was not tenable, since death was found to occur prior to crystallization (58).

Freezing was defined as removal of water from solution in the cellular matrix and its isolation into biologically inert ice crystals (35). This resulted in an increase in electrolyte concentration (31, 35, 58). According to Lovelock and others, exposure of cellular constituents to non-physiological levels of electrolyte concentrations within and outside the cell resulted in dissolution of the lipoprotein mantle of the cell wall and disruption of vital cellular organization (31, 35, 54, 58). A sudden change of osmotic pressure beyond the narrow tolerance limits during the extracellular crystallization caused osmotic shock, manifested in spermatozoa by an irreversible loss of motility (58). Electron microscopic studies substantiated the damage by temperature shock and osmotic changes (53).

Glycerol, a practical protective agent for storage of bovine spermatozoa at -79°C or -196°C (45, 49, 54, 56, 58, 62, 63), was shown to permeate the living cells in autoradiograph (47) and metabolic studies (33, 41, 43, 44, 54, 65, 68). Glycerol, a strong hydrogen binder, bound the water in the cell minimizing crystallization and dehydration preventing hypertonic concentration of salts (31, 35, 58). Microscopic studies indicated that glycerol promoted supercooling which postponed ice cormation until the temperature of the medium was lowered to a range of -10°C to -20°C. Extracellular ice crystals grew in fern like form leaving channels in which spermatozoa were lodged (58). Smith and Smiles (58) found no visible intracellular crystallization at temperatures as low as -80°C and no post-thaw morphological alteration in glycerol treated giant multinucleated spermatids of guinea pig testis.

Glycerol also was found to improve livability and fertility of liquid semen at 5°C in many diluents (5, 6, 7, 54, 64). Evidence has accumulated to show its utilization as a metabolite by the rat and sperm cells (33, 41, 43, 44, 54, 62, 65, 68).

Although glycerol has proved to be the best known cryogenic protective agent for sperm cells, certain limitations exist with it which call for explorations for better protective agents (16, 29, 35, 45, 46, 48, 54, 58, 62). These limitations and precautions are discussed in the following four paragraphs.

Addition of glycerol containing diluent to diluted semen in a single step at 5°C (6) or addition at above 5°C (6, 62) resulted in low survival rates. Consequently, addition of diluent containing glycerol dropwise with constant gentle stirring became a common practice (54).

Since the discovery of glycerol as a cryogenic protective agent for sperm cells, different levels of glycerol were advocated for optimum survivals. Recommended levels for bovine spermatozoa ranged from 4 percent to 20 percent for different extenders (3, 14, 21, 37, 45, 49, 50, 60, 62). There was closer agreement when egg yolk citrate was the diluent. These levels averaged 7 percent. Levels of glycerol higher than optimum were found to be toxic to sperm cells. Depressed livability, reduced progressive motility and irreversible immobilization were some of the effects observed with increasing levels of glycerol (29, 45, 56, 62). Higher levels of glycerol, due to its hydrogen binding characteristic, denatured proteins and thus became toxic (35).

Various equilibration times have been recommended for the freeze processing of bovine spermatozoa with glycerol. Motility and fertility studies indicated that 14.9 and 12 hours of glycerol equilibration was better than 4 hours (13, 23). The earlier recommendation of the British workers was a period of 15 to 20 hours (54). Shorter equilibration periods of 0 hours, 30 minutes, 4 hours, and 6 hours were favored over 18 hours in some of the reports (38, 40, 42, 62). Longer equilibration periods rendered commercial freezing operations cumbersome. However, in commercial freezing of semen, the emphasis was on a longer equilibration period of 12 to 18 hours which was a compromise between the optimum recommendation and the minimum time needed for the action of antibiotics, individual bull variations, and the lack of clarity about the optimum equilibration period (54, 69).

Stewart in his work with glycerol (60) reported a lower spermatozoan survival rate after freezing and thawing than that reported by the British workers. Recently Picket (48) reported that 40 percent of the spermatozoa were killed or immobilized during the freezing and thawing processes. Practical field results suggest that an average of 50 percent survival of sperm cells may be expected. It is possible that some other agent may improve survival rates.

Lovelock (30, 58) reported that the protective action of glycerol was due to its low molecular weight, ability to permeate freely into cells, miscibility with salt solutions and lack of toxicity. He postulated that other substances having similar properties might also prove satisfactory or superior to glycerol (30). A number of substances with the characteristics just described were tested for their cryogenic protective efficacy in the preservation of different types of cells (9, 15, 24, 30, 32, 39, 45, 49, 54, 55, 56, 58, 62, 63). Glycerol, ethylene glycol, propylene glycol, and diethylene glycol in concentrations of 24, 25, 30 and 30 percent respectively, were effective in preventing hemolysis during freezing and thawing of erythrocytes at all temperatures (30). Dimethyl sulfoxide was found to be effective in preventing hemolysis of bovine erythrocytes during freezing and thawing after 30 minutes equilibration as compared to failure with glycerol after an equilibration of two hours (32). Pyridine-N-oxide was reported to permeate erythrocytes at 3.2 M concentration, but failed to prevent hemolysis at -40°C and below. Dimethyl sulfoxide in concentration of 7.5 percent to 12.5 percent was reported to be superior to glycerol as a cryogenic protective agent in the preservation of mitochondria (24), mouse bone marrow cells (9), primary chick embryo fibroblasts (15), and thyroid glands (55).

The protective properties of many substances related to glycerol were investigated in the last decade for the preservation of spermatozoa at -79°C or -196°C, but neither details nor comparative studies were available in the majority of reports

(45, 49, 54, 56, 58, 62, 63). The conditions and media used for investigation of some of these agents were not suitable for long time storage of bovine spermatozoa (32, 45, 49, 56, 58). Except for a statement that no agent was equal to glycerol for low temperature preservation of semen (54, 62, 63), there were no references to comparative studies. A large number of polyhydric alcohols and their derivatives were tested for cryogenic protective properties. Among these agents, ethylene glycol and propylene glycol had effects comparable to glycerol in protecting avian and mammalian spermatozoa against damage at low temperatures (45, 49, 56, 58). No details about these studies were furnished. The medium used was Ringer's solution (45, 49). The percentage of sperm surviving after freezing was reported to be dependent on the basic extender and as well as the protective agent used (57). Sodium citrate was recommended in the freezing of semen to avoid the damage occurring in Ringer's solution (58). Ethylene glycol and propylene glycol in 6 percent concentrations were reported as having no effect on fertility of unfrozen semen (57). For rabbit spermatozoa, known to be sensitive to glycerol, 25 percent to 30 percent survival rates were reported after freezing in a medium containing 7.5 percent ethylene glycol (17). These reports suggest the possibility that ehtylene glycol and propylene glycol might prove equal to or better than glycerol as cryogenic protective agents when used in extenders suitable for long time storage of spermatozoa.

Lovelock and Bishop (32) reported that dimethyl sulfoxide had some cryogenic protective properties but was inferior to glycerol for the freezing of bovine spermatozoa (32). Concentrations as high as 15 percent were not toxic to spermatozoa (32). The freezing medium consisted of one part of egg yolk and 9 parts of 0.16 M sodium chloride solution with 0.1 percent glucose. The semen was first extended in egg yolk, then the dimethyl sulfoxide in sodium chloride solution was added in a single step (32). Bishop and Salisbury (10) reported that saline solution was unsuitable for the preservation of spermatozoa. Freezing procedures which involve either elimination of egg yolk in the protective agent fraction of the extender, or addition of protective agent to the extended semen in a single step were reported to result in low survival rates of spermatozoa (6, 26, 54). The nontoxic and fast permeating characteristics of dimethyl sulfoxide and its superiority to glycerol in protecting a variety of cells during freezing (9, 15, 24, 32, 55), suggest the possibility of dimethyl sulfoxide being more effective than glycerol if suitable procedures could be developed.

Unpublished data (18) at this station indicated that dimethyl sulfoxide in egg yolk citrate caused emulsification of fat globules, thus interfering with microscopic evaluation of semen. Thacker and Almquist (61) reported that motility estimation was facilitated by skim milk extenders as compared to whole milk, due to the absence of fat globules. Hence it was desirable to consider the use of nonfat dry milk (NFDM) powder reconstitute extender to facilitate evaluation of sperm motility. There was divergence of opinion in the literature with respect to optimum concentration of nonfat dry milk solids in the reconstituted extender and optimum level of glycerol for freezing bovine semen. Recommended levels ranged from 8.7 percent to 10 percent W/V for NFDM concentration (8, 14, 54, 66), and 4 percent to 11 percent V/V for glycerol concentration (8, 14, 54). Equilibration periods of 0, 0.5 and 4 hours were reported to be better than 18 hours for skim milk extenders (40, 42, 54). No optimum glycerol equilibration time was established for a NFDM extender.

In view of the foregoing, there was a need to establish optimum concentration of NFDM solids, optimum levels of glycerol and equilibration times for clarity in the commercial processing of bovine semen. After development of a NFDM extender that is comparable to yolk citrate glycerol, the NFDM glycerol extender could be used as a control for the study of the comparative efficacy of the various agents to avoid the use of two extenders.

EXPERIMENTAL PROCEDURE

Basic Design

In experiment I the semen supply was provided by a weekly collection of two or three ejaculates from each of four young Holstein bulls. Three young Holstein bulls were used for all other experiments. Since it has been shown that no significant differences existed between first and second ejaculates of a bull with regard to fertility (11) and percent survival after freezing (62), the ejaculates of each bull were mixed after individual evaluation for initial motility and volume and the weighted average values were used to determine the extension ratios. The concentration of spermatozoa in the mixed ejaculates was determined by a photolometer calibrated with a hemocytometer. Semen requirements were taken into account in determination of extension ratios. Extension ratios were calculated to provide a final concentration of from 25 to 30 x 10^6 progressively motile spermotozoa in experiment I and 10^7 per ml in all other experiments (38).

In regard to extenders, egg yolk citrate composition was one part egg yolk to four parts of 2.9 percent sodium citrate solution. Nonfat dry milk (NFDM) powder was reconstituted at the desired concentration with distilled water in a blender at 15,500 RPM for three minutes. The reconstitute was heated in a double boiler to 90° to 95°C and kept at that temperature for 10 minutes (4, 6, 52, 54, 61). The heated reconstitute was cooled to 70°F in a water bath and stored at 5°C for next day's use. Antibiotic additives consisted of 800 to 900 I.U. of penicillin and 800 to 900 micrograms of streptomycin per ml of all extenders (2, 54).

The following procedures were standard throughout this study:

1. The quantity of extender used for each treatment was 100 ml. The extender was divided into two equal parts and in one part the total quantity of the cryogenic protective agent was incorporated and designated as the protective agent fraction.

2. The semen, after evaluation, was extended in the extender fraction without protective agent.

3. The extended semen samples, the protective agent fractions, and the apparatus for adding the protective agent fraction were carried into the cold room at one time. Addition of protective agent fraction to the extended semen was done per drip (54) at an ambient temperature of 5°C. The drip apparatus consisted of a rotary stage with a detachable device to hold the sample flasks containing extended semen and a fixed funnel support above. Polysterene funnels with latex tubing attached to their stems, were fitted into the sockets of the funnel stand. This facilitated direct dripping of the protective agent fraction of the extender from the funnels into the sample flasks on the rotary stage. The flow through the latex tubing was regulated by screw clamps. The drip rate was adjusted to complete the addition of protective agent fraction in 30 minutes. The rotary stage provided gentle stirring of the extended semen during the addition of protective agent fraction. All samples from an individual bull were treated with their respective protective agent fractions at one time.

4. Equilibration periods of 6, 12 and 18 hours were used.

5. Test freezing of the samples was done in accordance

with the routine testing method developed by Paul L. Rempel, Laboratory Technician of Kansas Artificial Breeding Service Unit. Rempel's method was based on Graham's test freezing in thin films or breeding pipettes (22) and the technique of freezing in liquid nitrogen vapor by Forgason et al. (20).

The semen from each sample was drawn into a plastic breeding pipette of 16" length, 5 mm diameter and 1 ml capacity, and one end of it was sealed with a soldering iron. The sealed pipettes were pre-coded and introduced into styrofoam insulators of uniform size and their openings were closed with wooden plugs. At the end of an equilibration period, the insulated freeze samples were introduced into the nitrogen refrigerator and the frozen samples were stored at about -280°F (nitrogen vapor temperature).

6. Post freeze microscopic rating for percent progressively motile spermatozoa was accomplished after 24 to 48 hours of storage. The semen samples were evaluated in a random order by retrieving from the nitrogen refrigerator without reference to their pre-coding.

7. Thawing of the frozen samples in the styrofoam insulators, was done at room temperature. The thawed samples were examined within ten minutes after thawing on warm slides with cover slips at 100 X and 430 X magnifications.

8. Comparisons were based on percent survival.

9. A split sample design was used throughout the study because of its relative efficiency and freedom from sample to

sample variation (19). In accordance with the advice of Dr. S. Wearden, Statistical Consultant at this Experiment Station, duplicate samples were run within each equilibration time, bull and treatment. Each experiment was repeated three times, unless otherwise stated. Sample replicates and trial repetition facilitated the estimation of interaction and error components.

The design used in all the experiments involving freezing procedures is shown in Table 1.

A total of seven experiments, each consisting of one or more trials was conducted in this study. Two trials were conducted in experiment I to establish an optimum concentration of NFDM solids for a NFDM extender. Standard egg yolk citrate was used as a control. Grade A NFDM powder from Consolidated Badger Cooperative, Appleton, Wis. was used throughout the study. The powder was stored in a refrigerator at 5°C.

Since concentrations of NFDM powder ranging from 8.7 percent to 10 percent were found to be optimum by different workers (8, 14, 54, 66), 6, 10, and 14 percent W/V NFDM extenders both heated and unheated were compared to egg yolk citrate in trial one. Percent progressively motile spermatozoa was the comparison criterion. Based on the results of trial one, the range of 6 percent to 10 percent was further narrowed and different levels of heated NFDM extenders were compared to egg yolk citrate in trial two.

In all NFDM evaluation trials the extended semen samples

Table 1. Split sample design for freeze trials.

Equilibration	6 Hours				12 Hours				18 Hours				Total samples
Treatment Bull	Control	5%	7%	10%	Control	5%	7%	10%	Control	5%	7%	10%	
A	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	24
B	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	24
C	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	1 2	24
Total samples	6	6	6	6	6	6	6	6	6	6	6	6	72

were cooled at the rate of 1°F per 4 minutes and stored at 5°C in 5 ml capped plastic vials. The samples were evaluated every 24 hours for motility by microscopic examination for seven days.

Experiment II was designed to establish an optimum level of glycerol and equilibration time for freezing semen in the NFDM extender found to be optimum. In recent literature a range of 5 percent to 10 percent glycerol concentration was reported to be optimum. To determine optimum glycerol percentage more precisely three identical trials were conducted with 5, 7, and 10 percent glycerol V/V in NFDM extender. Standard egg yolk citrate with 7 percent glycerol V/V was used as control. The procedural steps for freezing and equilibration were as described previously.

Experiments III, IV, V, VI, and VII were conducted to study the cryogenic protective efficacy of ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide and pyridine-N-oxide, in concentrations of 5, 7 and 10 percent, using the same procedural steps of freezing and equilibration periods of experiment II. NFDM extender found to be optimum in experiment I was used as the basic medium, and glycerol in a concentration found to be optimum in experiment II was used as the control.

All data suitable for analysis were tested by analysis of variance and Fisher's Least Significant Difference (LSD) method was used for comparisons (59).

RESULTS AND DISCUSSION

Experiment I. Establishing optimum concentration of NFDM powder in a reconstitute extender.

Trial one. Trial one was conducted to establish an optimum concentration of NFDM powder in reconstitute extender within a range of 6 percent to 14 percent, and to test the effect of unheated NFDM extenders on sperm livability. Heated and unheated NFDM extenders with concentrations of 6, 10, and 14 percent solids were compared to a control of egg yolk citrate.

There were no progressively motile sperm present in the unheated NFDM extenders even on day one. The analysis of variance for the samples stored in heated NFDM extender is presented in Table 9 of the Appendix. There were highly significant ($P < 0.01$) differences among treatments on all days of storage. The average progressive motility for semen samples extended in heated NFDM extenders and egg yolk citrate (control) are presented in Table 2.

Information contained in Table 2 indicates that the 10 percent NFDM extender was equal to or better than control on all days. The control was better than 6 percent and 14 percent NFDM extenders. The 6 percent NFDM extender was equal to or better than 14 percent NFDM extender on all days. Visibility was uniformly good in NFDM extenders throughout the storage period and comparatively poor in the control from day four onward.

There was significant interaction ($P < 0.05$ on day one and $P < 0.01$ on rest of the days), between bulls and treatments

Table 2. Mean percent progressive motility of semen samples extended in 6, 10 and 14 percent NFDM extenders and yolk citrate control during a seven day storage at 5°C.

Extender	Days of Storage							Average motility
	1	2	3	4	5	6	7	
Egg yolk citrate	61.8^{b}[*]	52.5^{b}	45.0^{b}	35.6^{c}	23.4^{c}	12.5^{b}	7.8^{b}	34.1
6% NFDM	23.1^{a}	20.6^{a}	18.8^{a}	18.1^{b}	16.3^{b}	13.3^{b}	10.1^{b}	17.2
10% NFDM	65.0^{b}	56.3^{c}	52.5^{c}	44.4^{d}	33.1^{d}	16.3^{c}	10.1^{b}	39.7
14% NFDM	25.6^{a}	19.4^{a}	17.5^{a}	12.5^{a}	5.5^{a}	0.8^{a}	0.4^{a}	11.7
LSD ($P < 0.05$)	3.85	2.65	2.28	2.45	4.00	2.31	3.03	

[*] Means with common superscripts were not significantly different. Comparisons were among treatments within a day.

on all days. No LSD comparisons were made for this interaction for want of common error term for the entire storage period. Motility for semen of individual bulls in different extenders averaged over all days of storage are presented in Table 3. Ten percent NFDM extender was better than either 6 percent or 14 percent in all comparisons and superior to the control with the exception of bull A wherein the control was superior.

Table 3. Mean percent progressive motility for bulls and treatments averaged over seven days of storage at 5°C.

Bulls	Treatments			
	Control	6% NFDM	10% NFDM	14% NFDM
A	43	17	40	11
B	22	20	44	9
C	36	15	37	14
D	36	15	38	12

Trial two. The results of trial one revealed that the 10 percent NFDM extender was optimum in the 6 percent to 14 percent range. Interaction between bulls and treatments did not affect this inference. Trial two was conducted to establish the optimum concentration of NFDM powder in a further narrowed range of concentrations. Heated NFDM extenders with concentrations of 8, 9, 10, 11 and 12 percent solids were compared to a control of egg yolk citrate.

The analysis of variance for this trial is presented in Table 10 of the Appendix. The differences among treatments were highly significant ($P < 0.01$) on all days of storage. The mean progressive motility of semen samples in control and 8, 9, 10, 11 and 12 percent heated NFDM extenders are presented in Table 4.

The 10 percent NFDM extender was equal to or better than other concentrations on all days of storage and consistently superior to the control. The 8, 9, and 11 percent concentrations of NFDM were generally better than the control and 12 percent concentration. The control was better than 12 percent NFDM extender. Concentrations of NFDM below 10 percent were consistently superior to concentrations of NFDM above 10 percent.

There was significant interaction between bulls and treatments on days two, three, six ($P < 0.01$) and days four and five ($P < 0.05$). This interaction represents the deviation in the response of the individual bulls from the general trend of the results with different extenders and the inconsistency in the variations among the response of the bulls from treatment to treatment. However, 10 percent NFDM extender was better than the other extenders for all the bulls.

The results of trial one confirm the findings of previous workers that unheated milk extenders are toxic to sperm cells (54). The results of trials one and two showed that a range of 8-11 percent NFDM concentration was equal to or better than yolk citrate control on all days of storage. Ten percent NFDM extender was generally superior to 8, 9, or 11 percent NFDM

Table 4. Mean percent progressive motility of semen samples extended in a yolk citrate control and 8, 9, 10, 11 and 12 percent NFDM extenders on days two through seven of storage at 5°C.

Extender	Days of Storage						Average motility
	2	3	4	5	6	7	
Control	44.4^{ab*}	34.4^{a}	26.3^{bc}	16.3^{b}	9.4^{b}	4.9^{b}	22.6
8% NFDM	47.5^{b}	41.3^{b}	31.3^{cd}	17.5^{b}	13.1^{cd}	7.9^{c}	26.4
9% NFDM	52.5^{c}	43.1^{bc}	31.3^{cd}	18.8^{b}	12.5^{c}	9.0^{c}	27.9
10% NFDM	58.8^{d}	45.6^{c}	35.6^{d}	27.5^{c}	15.0^{d}	12.0^{d}	32.4
11% NFDM	51.9^{c}	40.0^{b}	25.6^{b}	13.8^{ab}	10.0^{b}	6.0^{bc}	24.5
12% NFDM	42.5^{a}	33.1^{a}	19.4^{a}	10.0^{a}	6.9^{a}	1.6^{a}	18.9
LSD ($P < 0.05$)	3.9	4.1	5.1	5.2	2.5	2.6	

*Means with common superscripts were not significantly different. Comparisons were among treatments within a day.

extenders and control. The range of 8-11 percent NFDM concentration tends to support previously reported optimum concentrations (5, 54, 66). However, a NFDM concentration of 10 percent was the optimum in this range, particularly in the first 48 to 72 hours of storage. Microscopic appearance of sperm cells was uniformly clear in NFDM extenders and motility estimation was easier than in egg yolk citrate due to the absence of fat globules. This is in accord with the report of Thacker and Almquist (61). When stored in bulk at 5°C the quality of NFDM powder remained good with respect to texture and absence of toxic substances during storage for three months. This, as was reported by Almquist and Flipse (5), demonstrated that NFDM powder was a reliable dry extender free of seasonal variation.

In both trials, extenders having concentrations of NFDM higher than optimum showed initial superiority. But, on storage, deterioration of motility was faster in extenders with concentrations above 10 percent. Like whole milk, higher concentrations of NFDM may provide higher levels of metabolites such as glucose (36, 54, 43) and unidentified carbohydrates (1, 54). The metabolic end-products of these metabolites may have lowered the pH of the medium as they accumulated. This in turn may have reduced the metabolic acitivity and motility of sperm cells (54).

From the results of this experiment it was apparent that a 10 percent W/V concentration of NFDM was the optimum for the survival of spermatozoa when stored at 5°C and better than the egg yolk citrate control under the conditions imposed.

Experiment II. Establishing optimum concentration of glycerol and equilibration time for 10 percent NFDM extender compared to egg yolk citrate with 7 percent glycerol, for freezing bovine semen.

This experiment was designed to establish an optimum glycerol concentration V/V and equilibration time in a 10 percent NFDM extender for freezing bovine semen. Three identical trials were conducted with 5, 7, and 10 percent concentrations of glycerol. Standard egg yolk citrate with 7 percent glycerol V/V was used as control.

The analysis of variance for this experiment is presented in Table 11 of the Appendix.

The differences among treatments, and equilibration periods, and the interaction between treatments and equilibration periods were highly significant ($P < 0.01$). The post-thaw survival rates of spermatozoa for treatments, equilibration periods, and the combinations of these two factors are presented in Table 5.

The results presented in Table 5 revealed that 7 percent glycerol in 10 percent NFDM extender was superior to the control and other treatments. The control was equal to NFDM with 5 percent glycerol and superior to NFDM with 10 percent glycerol. The post-thaw revival time of spermatozoa motility in samples frozen with 10 percent glycerol was relatively slow and progressive motility was weak. There was an overall inverse relationship

Table 5. Post-thaw survival rates of spermatozoa frozen in egg yolk citrate with 7 percent glycerol, and 10 percent NFDM extender with 5, 7, and 10 percent glycerol after 6, 12, and 18 hours equilibration periods.

Treatments	Equilibration in hours 6	12	18	Average for Treatment
7% glycerol in egg yolk citrate (control)	51.86[1]	42.17	37.26	43.8[2]
5% glycerol in 10% NFDM	55.31	47.93	43.92	49.1
7% glycerol in 10% NFDM	64.42	58.23	45.53	56.1
10% glycerol in 10% NFDM	44.76	25.78	12.62	27.7
Average for equilibration	54.1[3]	43.5	34.8	

[1] LSD ($P < 0.05$) for comparisons among equilibration periods within a treatment = 11.966

[2] LSD ($P < 0.05$) for comparisons among averages for treatments = 6.9

[3] LSD ($P < 0.05$) for comparisons among averages for equilibration periods = 5.887

between hours of equilibration and survival rates. A 6 hour equilibration period was superior to 12 and 18 hours.

The interaction between treatments and equilibration periods represents the significant deviations of treatment and equilibration period combinations from the inferences about treatments and equilibration periods. A 7 percent glycerol in 10 percent NFDM extender with an equilibration period of 6 hours was optimum. Results of additional studies with 7 percent glycerol in 10 percent NFDM extender revealed that 0 and 3 hour equilibration periods with survivals of 33 and 35 percent were not better than a longer equilibration period of 6 to 18 hours.

There were highly significant ($P < 0.01$) interactions between bulls and treatments, and bulls and equilibration periods. These interactions represent the inconsistency in the variations among the bulls with each treatment, and equilibration period. However, with all bulls 7 percent glycerol in 10 percent NFDM and 6 hour equilibration periods were optimum.

The results of this experiment pertaining to levels of glycerol are in agreement with the findings of Jones *et al.* (29), Curtis *et al.* (14), Miller and Van Demark (38) and Van Demark *et al.* (63). Although different concentrations of NFDM with different concentrations of glycerol were not tested in this experiment, the general trend of the results contradict the findings of Amann and Almquist (8). The results of experiments I and II agree with the prediction that a 10 percent NFDM would be optimum for freezing bovine semen (8); but the results of experiment II contradict the prediction that a 9.7 percent glycerol V/V in 10 percent NFDM extender would be optimum (8) for freezing semen.

An equilibration period of 6 hours was consistently superior to 12 and 18 hours in this experiment. This is in agreement with the findings of some earlier workers (37, 38, 63). With 7 percent glycerol in egg yolk citrate, an equilibration period of 6 hours was superior and the differences between 6 and 12 hours were not significant. This is a new finding since the previous workers have not compared 6 hours with 12 hours (37, 38, 62) and 18 hours (13, 23) in one experiment. When summarized as a group the findings of previous workers (13, 23, 37, 38, 62) and this experiment suggest a range of 6 to 12 hours equilibration as optimum, with slight advantage for 6 hours. An equilibration of 6 hours was better for 7 percent glycerol in 10 percent NFDM extender, than 12 and 18 hours. There were no significant differences between 6 and 12 hours of equilibration. Equilibration periods of 0 and 3 hours had no advantage over longer equilibration periods for 7 percent glycerol in 10 percent NFDM extender. The discrepancy between this finding and those of O'Dell and Hurst (40) and O'Dell and Almquist (42) might be due to higher concentrations of glycerol used in their studies. The clear inverse relationship between equilibration time and survival in this experiment with 10 percent glycerol is in agreement with the trend of the findings of these workers (40, 42), and it is apparent that with concentrations of glycerol higher than optimum, shorter equilibration periods are better.

Experiment III. Cryogenic protective efficacy of ethylene glycol compared to glycerol.

In this experiment three identical trials were conducted to study the protective efficacy of ethylene glycol at concentrations of 5, 7, and 10 percent V/V when compared to 7 percent glycerol V/V in a basic medium of 10 percent NFDM extender. In trial two the samples were examined for prefreezing motility after a 6 hour equilibration period.

The analysis of variance for this experiment is presented in Table 12 of the Appendix.

The differences among treatments, and equilibration periods, and the interaction between treatments and equilibration periods were highly significant ($P < 0.01$). The post-thaw survival rates of spermatozoa for treatments, equilibration periods, and combinations of treatments and equilibration periods are presented in Table 6.

The results presented in Table 6 revealed that the control was superior to other treatments. The 5 percent ethylene glycol extender was superior to 7 percent and 10 percent ethylene glycol and 7 percent ethylene glycol was superior to 10 percent ethylene glycol. A 6 hour equilibration period was superior to 12 and 18 hour equilibration periods.

The interaction between treatments and equilibration periods represents the significant deviations of treatment and equilibration period combinations from the inferences about treatments and equilibrations. However, a 6 hour equilibration period was optimum for 7 percent glycerol and 5 percent ethylene glycol.

Average pre-freezing motilities for the control, and 5, 7, and 10 percent ethylene glycol were 60, 53.3, 41.6, and

Table 6. Post-thaw survival rates of spermatozoa frozen in 10 percent NFDM extender with 7 percent glycerol and 5, 7, and 10 percent ethylene glycol after 6, 12, and 18 hours equilibration periods.

Treatments	Equilibration in hours			Average for Treatment
	6	12	18	
7% glycerol in 10% NFDM (control)	58.2[1]	42.4	40.5	47.0[2]
5% ethylene glycol in 10% NFDM	41.9	29.9	14.9	28.9
7% ethylene glycol in 10% NFDM	25.6	17.6	10.5	17.9
10% ethylene glycol in 10% NFDM	5.6	5.2	1.7	4.1
Average for equilibration	32.8[3]	23.7	16.9	

[1] LSD ($P < 0.05$) for comparisons among equilibration periods within a treatment = 4.46

[2] LSD ($P < 0.05$) for comparisons among averages for treatments = 5.15

[3] LSD ($P < 0.05$) for comparisons among averages for equilibration periods = 8.94

36.6 percent respectively. The pre-freezing motility of spermatozoa in 7 and 10 percent ethylene glycol was abnormal as evidenced by oscillatory and circular motility.

There were significant interactions between bulls and treatments ($P < 0.05$), and bulls and equilibration periods ($P < 0.01$). These interactions represent the inconsistency in the variations among the bulls with individual treatments, and equilibration periods. With all bulls 7 percent glycerol and 6 hours equilibration period were superior.

Since ethylene glycol was lower in molecular weight than glycerol and revival time for semen samples frozen in ethylene glycol was directly proportional to the concentrations used and the length of equilibration time, one additional trial was conducted to study the efficacy of ethylene glycol at concentrations of 1, 3 and 5 percent V/V and shorter equilibration periods of 0, 3 and 6 hours. The results of this trial revealed that the control had an average survival of 45.4 percent and was better than 1, 3 or 5 percent ethylene glycol with 3.3, 23.2, and 32.7 percent survival respectively.

With the control, equilibration periods of 0 and 3 hours resulted in lower survival rates (26.6 and 44.3 percent) than 6 hour equilibration which were 65.3 percent survival. The survival rates with 1 percent ethylene glycol were negligible at any equilibration period. With 3 percent ethylene glycol, 3 hours equilibration period with 27.9 percent survival was better than 0 and 6 hour equilibration periods with 17.1 and 24.7 percent survival. With 5 percent ethylene glycol, 3 hours equilibration period with a survival of 41.5 percent was better than 0 and 6 hours equilibration periods with 24.3 and 32.5 percent survival.

The results of this experiment showed that ethylene glycol was less efficacious than glycerol in its protective ability for bovine spermatozoa. This is in agreement with the previous reports (49, 56, 58). A 5 percent concentration of ethylene glycol was the optimum for freezing bovine semen. Concentrations of ethylene glycol above 5 percent were toxic for sperm cells. This was evident from the fact that the prefreezing motilities with 7 and 10 percent ethylene glycol were predominantly oscillatory and circular. It was not determined whether this toxicity of ethylene glycol was due to its binding characteristic as in the case of 10% glycerol (35, 62); or its hypertonicity and damage to the structure of sperm cell (53); or its uncoupling of oxidative phosphorylation (65); or the oxidative conversion of ethylene glycol to oxalic acid and intermediary products like glyoxal and glyoxylic acid (58, 68) and the resultant toxicity due to the limited capacity of sperm cells to detoxify end products (54). This, however, is a matter that should be further investigated. Ethylene glycol concentrations of less than 5 percent were not as effective as 5 percent concentration in the protection of sperm cells against freezing. The fact that 5 percent ethylene glycol, with 3 hours of equilibration, resulted in higher survival of sperm cells than either 0 or 6 hour equilibration periods and 7 percent glycerol resulted in higher survival with a 6 hour equilibration period compared to either 0 or 3 hours, suggests that ethylene glycol might permeate sperm cells quicker than glycerol. The lower efficacy of ethylene glycol compared to glycerol in spite of its fast permeation of sperm cells, low molecular weight, high solubility in aqueous electrolyte solution, and lack of toxicity at 5 percent concentration (58) suggest, that some character other than these might be lacking. A protective agent to be efficient might also need to be a metabolite for sperm cells. The reports that ethylene glycol at a concentration of 7.5 percent with 1.25 percent or more of any monosaccharide protected rabbit spermatozoa better than glycerol during freezing (17), and glycerol was metabolized by sperm cells (33, 41, 44, 65), support this. Ethylene glycol which is not a nutrient (68) might permeate sperm cells to the exclusion of other metabolites in the extender during equilibration due to its smaller molecular weight (34). Sperm metabolism goes on at 5°C or during storage at freezing temperatures although at a lower rate (54). The limited intracellular sources of energy in sperm cells might be exhausted even during equilibration and this might result in low survival after thawing. In contrast, glycerol, being a metabolite, is probably used by sperm cells and hence higher survivals might result. Combinations of glycerol or any sugar with ethylene glycol might prove superior to glycerol and conserve time in freezing operations.

An equilibration period of 6 hours that was optimum for ethylene glycerol, followed by 12 and 18 hours, indicates that shorter equilibration periods are better than longer ones. Results of trial 4 suggest that a 3 hour equilibration period is optimum for 5 percent ethylene glycol.

The presence of significant interactions between bulls and treatments, and bulls and equilibrations in this experiment concur with the findings of Graham (22), but these interactions did not alter the inferences about treatments and equilibrations since they represent the inconsistency in the variations among bulls with individual treatments and equilibration periods.

Experiment IV. Cryogenic protective efficacy of propylene glycol compared to glycerol.

In this experiment the protective efficacy of propylene glycol at concentrations of 5, 7, and 10 percent V/V compared to 7 percent glycerol V/V in a basic medium of 10 percent NFDM extender was studied. Owing to the negligible results obtained, the experiment was conducted only once. Samples were examined for pre-freezing motility after a 6 hour equilibration period.

The post-thaw survivals of spermatozoa with the control at 6, 12, and 18 hours equilibration periods were 48.4, 32.3 and 20.9 percent respectively. The post-thaw survivals with propylene glycol in concentrations 5, 7, and 10 percent were 0.9, 0.4, and 0.0 percent respectively. The pre-freezing motilities for the control and 5 and 7 percent of propylene glycol were 63 percent; and for 10 percent propylene glycol it was 58.3 percent. The pre-freezing motility of sperm cells with 5 and 7 percent propylene glycol compared well with that of control, both in character and intensity; while with 10 percent propylene glycol it was predominantly oscillatory and weak.

The results of this experiment revealed that propylene glycol had no appreciable cryogenic protective ability for bovine spermatozoa. This apparent absence of protective ability is not in agreement with the reports of previous workers (49, 56, 57, 58). However, no details were furnished in those reports, except for a brief statement that it was less protective than glycerol. The facts that propylene glycol was metabolized by the rat, rabbit, and cat, and the pre-freezing motilities in concentrations of 5 and 7 percent were qualitatively and quantitatively equal to the control in this experiment, do not support the conclusion that its poor cryogenic protection for sperm cells was due to its toxicity (49, 58). Its apparent lack of cryogenic protection in this experiment was not surprising, since conflicting opinions were expressed in a single report about its efficacy in preventing the hemolysis during freezing and thawing of erythrocytes (30). Its lack of toxicity to sperm cells at 5°C and its smaller molecular size than glycerol, suggest that its failure as a cryogenic protective agent might be due to its poor solubility in aqueous electrolyte solutions.

Experiment V. Cryogenic protective efficacy of diethylene glycol compared to glycerol.

This experiment was designed to study the protective efficacy of diethylene glycol at 5, 7 and 10 percent levels V/V compared to 7 percent glycerol V/V in a basic medium of 10 percent NFDM extender. No repetition of this experiment was made due to the relatively poor protection observed compared to glycerol. Samples were examined for pre-freezing motility after 6 hours of equilibration.

The post-thaw survival rates of spermatozoa in the control and 5, 7, and 10 percent diethylene glycol were 37.7, 6.6, 5.1 and 0.8 percent respectively. The pre-freezing motilities in the control, and 5, 7 and 10 percent diethylene glycol were 53.3, 50, 46.6 and 41.6 percent respectively.

Diethylene glycol, based on pre-freezing motility, appeared to be non-toxic to sperm cells at 5°C. This was expected since it was less toxic to the rat than ethylene glycol (68). However, it did not appear to provide cryogenic protection for bovine spermatozoa. The apparent lack of toxicity at 5°C and the negligible survival rates after freezing indicated that its failure as a protective agent might be due to its poor permeation into sperm cells due to its larger molecular size than glycerol and lack of nutrient characters. Its effectiveness in preventing hemolysis during freezing and thawing of erythrocytes (30) and failure to protect sperm cells during freezing, supports the postulation that the usefulness of a cryogenic protective agent may also depend on the type of cells frozen (35).

Experiment VI. Cryogenic protective efficacy of dimethyl sulfoxide compared to glycerol.

In this experiment the protective efficacy of dimethyl sulfoxide at 5, 7 and 10 percent levels V/V compared to 7 percent glycerol V/V in a basic medium of 10 percent NFDM extender was studied. Three identical trials were conducted under this experiment. Samples were examined for pre-freezing motility after a 6 hour equilibration period.

The analysis of variance for this experiment is presented in Table 13 of the Appendix.

The differences among treatments, and equilibration periods were highly significant ($P < 0.01$). The post-thaw survival rates of spermatozoa for treatments, and equilibration periods are presented in Table 7.

Table 7. Post-thaw survival rates of spermatozoa frozen in 10 percent NFDM extender with 7 percent glycerol, and 5, 7, and 10 percent dimethyl sulfoxide after 6, 12, and 18 hours equilibration periods.

Treatments	Equilibration in hours			Average for Treatment
	6	12	18	
7% glycerol in 10% NFDM (control)	61.6	51.3	43.5	52.1[1]
5% dimethyl sulfoxide in 10% NFDM	18.1	17.9	16.7	17.6
7% dimethyl sulfoxide in 10% NFDM	21.0	18.9	10.7	16.8
10% dimethyl sulfoxide in 10% NFDM	5.9	2.5	3.4	3.9
Average for equilibration	26.6[2]	22.6	18.5	

[1] LSD ($P < 0.05$) for comparisons among averages for treatments = 7.7

[2] LSD ($P < 0.05$) for comparisons among averages for equilibration periods = 6.7

The results presented in Table 7 revealed that the control was superior to other treatments. A concentration of 5 or 7 percent dimethyl sulfoxide and an equilibration period of 6 or 12 hours were optimum for freezing bovine semen. The survival rates with 10 percent dimethyl sulfoxide were negligible. Results of additional observations revealed that with dimethyl sulfoxide, the survival rates of spermatozoa after an equilibration of 0 and 3 hours were lower than the survival rate after an equilibration of 6 hours.

The pre-freezing motilities of the control, 5, 7, and 10 percent dimethyl sulfoxide were 58.3, 56.6, 56.6 and 55 percent respectively. The motility was progressive in all extenders. Since the lower survival of spermatozoa with dimethyl sulfoxide may have been due to oxidation of the sulfhydryl group containing enzymes and proteins of sperm, a combination of 7 percent dimethyl sulfoxide and 1 in 5000 concentration of thioglycolic acid (28) was examined for its protective value. In this case the semen was extended into the whole extender at room temperature, then cooled and frozen. This resulted in a survival of 34.7 percent at 6 hour equilibration period which was better than the 21 percent survival with 7 percent dimethyl sulfoxide under usual freezing procedures.

Results of this experiment showed that dimethyl sulfoxide, a better protective agent than glycerol for freezing a variety of cells (9, 15, 24, 32, 55), was less efficacious than glycerol for protecting bovine sperm cells in this experiment. This is in agreement with the finding of Lovelock and Bishop (32). The higher survival rates obtained with a combination of dimethyl sulfoxide and low concentration of thioglycolic acid suggest that the lower cryogenic protection afforded by dimethyl sulfoxide alone compared to the addition of thioglycolic acid might be due to the oxidation of sulfhydryl group containing proteins and cellular enzymes of sperm cells. Obviously thioglycolic acid may have counteracted the action of dimethyl sulfoxide by producing an intensely reduced medium (54).

Absence of any significant difference between 6 and 12 hours equilibration periods and the lower survival rates at 0 and 3 hours equilibrations with dimethyl sulfoxide indicates that it did not permeate sperm cells quicker than glycerol. This is not in agreement with the finding of Lovelock and Bishop (32).

Experiment VII. Cryogenic protective efficacy of pyridine-N-oxide compared to glycerol.

In this experiment the protective efficacy of pyridine-N-oxide at 5, 7, and 10 percent levels W/V compared to 7 percent glycerol V/V in a basic medium of 10 percent NFDM extender was studied. The experiment was not repeated due to the relatively poor results obtained. Samples were examined for pre-freezing motility after 6 hours of equilibration.

The pre-freezing motility was 53.3 percent for the control and 5 percent pyridine-N-oxide, and 50 percent for

7 and 10 percent pyridine-N-oxide. The motility of the samples was sluggish and oscillatory. The post-thaw survival rate for the control was 44.5 percent. The post-thaw survival rates with 5, 7 and 10 percent pyridine-N-oxide were 0.8, 4.6, and 7.8 percent respectively with sluggish motility.

The character of pre-freezing motility of spermatozoa in pyridine-N-oxide samples suggests that pyridine-N-oxide was toxic to sperm cells. The negligible post-thaw survival rates suggest that pyridine-N-oxide did not possess any appreciable cryogenic protective ability. This is in accord with the finding of Nash in freezing and thawing of erythrocytes (39).

Comparative Efficacy. The post-thaw survival rates of spermatozoa frozen with glycerol, ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide, and pyridine-N-oxide at their respective optimum concentrations in a medium of 10 percent NFDM extender, after equilibrations of 6, 12, and 18 hours are presented in Table 8.

Results presented in Table 8 revealed that none of the protective agents used in this study afforded protection equal to that of glycerol with any equilibration period observed. The protection afforded by propylene glycol, diethylene glycol, and pyridine-N-oxide was not appreciable. Ethylene glycol was superior to dimethyl sulfoxide with any equilibration period. A 5% ethylene glycol with a 6 hour equilibration was superior to other protective agents.

Table 8. Post-thaw survival rates of spermatozoa frozen with 7 percent glycerol, 5 percent ethylene glycol, 5 percent propylene glycol, 5 percent diethylene glycol, 7 percent dimethyl sulfoxide, and 10 percent pyridine-N-oxide in 10 percent NFDM extender after equilibrations of 6, 12, and 18 hours.

Treatment	Equilibration in hours			Average for Treatment
	6	12	18	
7% glycerol in 10% NFDM (control)	64.42	58.23	45.53	56.1
5% ethylene glycol in 10% NFDM	41.9	29.9	14.9	28.9
5% propylene glycol in 10% NFDM	2.1	0.6	0.0	0.9
5% diethylene glycol in 10% NFDM	11.6	2.7	5.7	6.6
7% dimethyl sulfoxide in 10% NFDM	21.0	18.9	10.7	16.8
10% pyridine-N-oxide in 10% NFDM	12.6	6.6	4.2	7.8

Reliability of Rempel's method of Test Freezing. The differences between replicates across all trials, bulls, equilibrations, and treatments involving a total of 1080 observations, were found to be not significant when the data was subjected to the "t" test ($P < 0.05$). This lack of significance suggests that Rempel's method of test freezing was reasonably reliable, for evaluation of studies involving freezing procedures.

SUMMARY AND CONCLUSIONS

The cryogenic protective efficacy of ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide, and pyridine-N-oxide compared to glycerol, for the preservation of bovine spermatozoa in a basic medium of NFDM extender at about -280°F, was investigated.

The results of Experiment I showed that unheated NFDM extenders were toxic to sperm cells. A range of 8 to 11 percent W/V NFDM concentration was suitable for the survival of spermatozoa and storage at 5°C. The optimum was with a 10 percent W/V concentration and was better than egg yolk citrate. Microscopic appearance of sperm cells was clear, and motility estimation easier in NFDM extenders than with yolk citrate. When stored at 5°C the quality of NFDM powder remained good with respect to texture and development of toxic substances during storage for three months. Extenders having higher concentrations of NFDM than optimum showed initially superiority to lower concentrations, but on storage deterioration was faster in extenders with concentrations above 10 percent.

Results of Experiment II indicated that a concentration of 7 percent glycerol was optimum in 10 percent NFDM extender for freezing bovine semen and better than egg yolk citrate with 7 percent glycerol. An equilibration period within a range of 6 to 12 hours appeared to be optimum for 7 percent glycerol in egg yolk citrate and 10 percent NFDM extenders, with slight superiority for 6 hours. Equilibration periods of 0 and 3 hours had no advantage over a range of 6 to 18 hours in the case of 7 percent glycerol in NFDM extender. A 10 percent glycerol concentration was toxic to sperm cells.

Experiment III revealed that ethylene glycol was less efficacious than glycerol as a cryogenic protective agent for sperm cells. A 5 percent ethylene glycol at an equilibration period of 3 hours was optimum for freezing bovine spermatozoa. The 7 and 10 percent concentration of ethylene glycol were toxic to sperm cells. The cause of this toxicity was not determined. The results indicated that ehtylene glycol might be permeating sperm cells faster than glycerol and its comparatively lesser efficacious cryogenic protection might be due to its being not useful to sperm cells as a nutrient. Combinations of ethylene glycol and either glycerol or any sugar might prove superior to glycerol and conserve time in freezing operations.

Results of Experiment IV indicated that propylene glycol in concentrations of 5 and 7 percent was not toxic to sperm cells at 5°C. The cryogenic protection afforded to sperm cells by this substance was negligible. Its failure as a cryogenic protective agent to sperm cells may have been due to its poor solubility in aqueous electrolyte solutions.

Experiment V revealed that diethylene glycol was not toxic to sperm cells at 5°C. This substance did not possess any appreciable cryogenic protective efficacy to sperm cells and the failure might be due to its poor permeating ability and lack of any nutrient characters.

The results of Experiment VI showed that dimethyl sulfoxide was less efficacious than glycerol in protecting sperm cells against freezing damage, with indications that this might be due to the oxidation of sulfhydryl group containing proteins and cellular enzymes of sperm cells by dimethyl sulfoxide. Combinations of substances like thioglycolic acid or cysteine hydrochloride with dimethyl sulfoxide might provide better protection than dimethyl sulfoxide alone. Dimethyl sulfoxide did not appear to permeate sperm cells faster than glycerol. Concentrations of 5 and 7 percent dimethyl sulfoxide were optimum for freezing bovine semen at 6 or 12 hours equilibration period.

Experiment VII showed that concentrations of 5, 7 and 10 percent pyridine-N-oxide were toxic to sperm cells at $5^{\circ}C$ and did not possess any appreciable cryogenic protective ability for sperm cells.

None of the cryogenic protective agents tested in this study provided protection to sperm cells equal to that of glycerol. Ethylene glycol and dimethyl sulfoxide were the only substances which had appreciable protective capacity. Ethylene glycol was superior to dimethyl sulfoxide with any equilibration period.

Based on the criteria of pre-freezing progressive motility, and post-thaw survival of spermatozoa under the conditions imposed in this study the following conclusions can be drawn:

(1) A 10 percent NFDM extender was superior to egg yolk citrate for the preservation of bovine semen at $5^{\circ}C$.

(2) A 10 percent NFDM extender with 7 percent glycerol was superior to egg yolk citrate with 7 percent glycerol for the preservation of bovine semen at about $-280^{\circ}F$.

(3) An equilibration period of 6 to 12 hours was optimum for freezing bovine spermatozoa with 7 percent glycerol in either 10 percent NFDM extender or egg yolk citrate.

(4) Rempel's method of test freezing appeared to be a reasonably reliable technique for the evaluation of various freeze procedures.

(5) The cryogenic protective efficacy of ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide and pyridine-N-oxide which were tested in this study for spermatozoa was less than that of glycerol. The failure of many substances except that of pyridine-N-oxide did not appear to be due to toxicity. Further fundamental studies seem to be indicated in order to assess further the requisite properties of a cryogenic protective agent for bovine spermatozoa.

(6) Further studies are recommended using combinations of ethylene glycol and either glycerol or a monosaccharide, and dimethyl sulfoxide and either thioglycolic acid or cysteine hydrochloride.

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APPENDIX

Table 9. Analysis of variance for Trial one of Experiment I.

Source	Degrees of Freedom	Mean Squares Day 1	2	3	4	5	6	7
Bulls	3	90.3**	7.8	9.3	5.3	43.6	6.0	56.3**
Treatments	3	4090.0**	3171.0**	2578.2**	1769.3**	1086.0**	372.3**	170.6**
Bull X Treatments	9	37.0*	44.2**	70.5**	115.3**	171.1**	101.5**	76.1**
Error	16	13.3	6.3	4.7	5.4	14.4	4.8	8.2
Total	31							

* F ratio significant ($P < 0.05$)

** F ratio significant ($P < 0.01$)

Table 10. Analysis of variance for Trial two of Experiment I.

Source	Degrees of Freedom	Mean Squares					
		Day 2	3	4	5	6	7
Treatments	5	287.0**	193.4**	259.2**	277.0**	69.2**	100.4**
Bulls	3	220.6**	168.0**	238.0**	50.6	175.0**	269.0**
Treatments X Bulls	15	46.3**	73.0**	65.1*	29.0	26.8*	18.4*
Error	24	14.5	15.6	24.5	26.0	5.7	6.6
Total	47						

* F ratio significant ($P < 0.05$)

** F ratio significant ($P < 0.01$)

Table 11. Analysis of variance for Experiment II.

Source	Degree of Freedom	Sum of Squares	Mean Squares	F
Bulls	2	233.9	111.9	1.0687
Treatments	3	23547.5	7849.1	74.967**
Equilibrations	2	13389.7	6694.8	63.942**
Bulls X Treats.	6	2948.9	491.4	4.6934**
Treats. X Equilib.	6	2533.8	422.3	4.0334**
Bulls X Equilib.	4	3272.1	818.0	7.812**
Bulls X Treats. X Equilib.	12	176.6	14.7	0.1404
Error	36	3770.5	104.7	
Total	71	49863.0		

** F ratio significant ($P < 0.01$)

Table 12. Analysis of variance for Experiment III.

	Degrees of Freedom	Sum of Squares	Mean Square	F
Bulls	2	2042.0	1021.0	17.51**
Treatments	3	53093.3	17697.7	303.5**
Equilibrations	3	9152.3	4576.1	78.49**
Bulls X Treatments	6	892.2	148.7	2.55*
Bulls X Equilibrations	4	1214.5	303.6	5.21**
Equilibrations X Treats.	6	3009.9	501.6	8.60**
Bulls X Equilibrations X Treats.	12	463.6	38.6	0.66
Error	36	2101.1	58.3	
Total	71	71968.9		

* F ratio significant ($P < 0.05$)

** F ratio significant ($P < 0.01$)

Table 13. Analysis of variance for Experiment VI.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Treatments	3	69068.5	23022.8	168.54**
Equilibrations	2	2363.6	1181.8	8.65**
Bulls	2	1813.8	906.9	6.64**
Bulls X Treatments	6	3885.0	647.5	4.74**
Equilibrations X Treats.	6	1844.1	307.3	2.25
Bulls X Equilibrations	4	517.1	129.2	0.95
Treats. X Bulls X Equilibrations	12	921.7	76.8	0.56
Error	36	4920.1	136.6	
Total	71	85333.9		

** F ratio significant ($P < 0.01$)

COMPARATIVE EFFICACY OF SELECTED CRYOGENIC PROTECTIVE AGENTS FOR THE PRESERVATION OF BOVINE SPERMATOZOA

by

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Seven experiments were conducted to investigate the cryogenic protective efficacy of ethylene glycol, propylene glycol, diethylene glycol, dimethyl sulfoxide, and pyridine-N-oxide compared to glycerol, for the preservation of bovine spermatozoa at about -280°F, in a basic medium of nonfat dry milk (NFDM) extender.

A split sample design with duplicate samples within each equilibration period, bull, and treatment was used throughout this study. Equilibration periods of 6, 12, and 18 hours were used throughout. Freezing of the samples was accomplished in accord with Rempel's method of test-freezing.

Experiment I was used to establish an optimum concentration of NFDM powder in a reconstituted extender for the preservation of bovine semen at 5°C, compared to egg yolk citrate. Experiment II was used to establish a control of optimum glycerol concentration in NFDM extender, compared to egg yolk citrate with 7 percent glycerol, for freezing bovine spermatozoa. Experiments III, IV, V, VI, and VII were conducted to investigate the cryogenic protective efficacy of the aforesaid substances for bovine spermatozoa, in concentrations of 5, 7, and 10 percent compared to glycerol in a basic medium of NFDM extender.

The following were the significant observations in this study:

(1) A 10 percent NFDM concentration was optimum with respect to livability of bovine spermatozoa on storage at 5°C, and significantly better ($P < 0.05$) than egg yolk citrate; (2)

7 percent glycerol in 10 percent NFDM extender was optimum for freezing bovine spermatozoa with a post-thaw survival of 56.1 percent, and significantly better ($P < 0.05$) than 7 percent glycerol in egg yolk citrate with a post-thaw survival of 43.8 percent; (3) a 6 to 12 hour equilibration was optimum for 7 percent glycerol in either egg yolk citrate or 10 percent NFDM extender with a superiority which was not significant, for 6 hour equilibration; (4) ethylene glycol was less efficacious than glycerol ($P < 0.05$) in protecting spermatozoa during freezing; (5) propylene glycol, in concentrations below 10 percent, was not toxic to sperm cells at 5°C, but the cryogenic protection was negligible; (6) diethylene glycol was not toxic to sperm cells at 5°C, but demonstrated little cryogenic protection; (7) dimethyl sulfoxide was less efficacious than glycerol ($P < 0.05$) in protecting sperm cells against freezing damage; and (8) pyridine-N-oxide was toxic to sperm cells at 5°C, and therefore not suitable as a protective agent.

None of the substances tested in this study provided cryogenic protection for bovine spermatozoa equal to that of glycerol. Ethylene glycol and dimethyl sulfoxide afforded appreciable cryogenic protection. Ethylene glycol was superior to dimethyl sulfoxide in its protective efficacy. The failure of propylene glycol and diethylene glycol did not appear to be due to toxicity.

Further fundamental studies seem to be necessary in order to reassess the requisite properties of a cryogenic protective agent for bovine spermatozoa.

Rempel's method of test-freezing appeared to be reasonably reliable for evaluation of studies involving freeze procedures.